Exhibit M

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CHAPTER 24

Protein production in transgenic animals

Yann Echelard and Harry M. Meade

GTC Biotherapeutics, Inc, Five Mountain Road, Framingham, MA 01701, USA
Tel.: (508) 370-2420; fax: (508) 370-5299. E-mail: yann.echelard@gtc-bio.com

1. Introduction

Since insulin became available for the treatment of diabetes in 1922, protein therapeutics have been derived from fluids or tissues of animal origin. The biotechnology and genomic revolutions have led to the identification of an increasing number of human proteins and their development for therapeutic uses. Concurrently, the realization during the 1980s of the consequences of the use of human blood or tissue sources contaminated with adventitious infectious agents has provided added incentives for the development of recombinant production strategies for human polypeptides. A number of methods are employed to manufacture recombinant proteins for pre-clinical and clinical uses. Bioreactors using bacterial, yeast and mammalian cell cultures are routinely used for production of approved protein therapeutics. However, there is a need for alternative methods. For example, cost-effective recombinant production of complex molecules such as antithrombin, α_1-antitrypsin, or serum albumin, all used in high doses and currently extracted from human plasma, has not yet been achieved in microbial or mammalian cell bioreactors. In addition, since capital investments associated with production plants represent a significant portion of the development cost of new recombinant drugs, the inherent risk associated with the regulatory approval process is another stimulus for the development of flexible and inexpensive approaches for the manufacture of therapeutic proteins.

The introduction of foreign DNA, first into the mouse [1] and later into the livestock genome [2], as well as the characterization of tissue-specific regulatory sequences have provided the opportunity to express a wide range of proteins of biopharmaceutical interest in farm animals. An expression vector, comprising a gene encoding the human or humanized target protein fused with tissue-specific regulatory sequences, is inserted into the germline of the selected production species. When integrated, the expression construct becomes a dominant genetic characteristic that is inherited by the progeny of the founder animal. This general strategy makes it possible to harness the ability of animal tissues to produce large quantities of complex proteins. It has been utilized to target expression of therapeutic proteins to the milk, blood, urine and even the seminal fluids of various species. In addition, there is intense research on the targeted expression of foreign proteins to chicken eggs. Among these transgenic animal production systems, the transgenic mammary gland is the most advanced. Although most of this chapter will be dedicated to reviewing this system, the strategies and challenges relevant to the production of recombinant proteins in the milk of transgenic animals are largely applicable to other systems.

2. Production of recombinant proteins in the milk of transgenic animals

2.1. General overview

Following the first descriptions of the targeting of heterologous proteins to the mammary gland of transgenic mice [3–5] there was a flurry of articles reporting the generation of transgenic sheep, goats, cows and pigs carrying milk-specific transgenes (reviewed in Refs. [6–9]). Production of human recombinant protein pharmaceuticals in the milk of transgenic farm animals solves many of the problems associated with either microbial or animal cell expression systems. Bacteria often improperly fold complex proteins, leading to involved and expensive refolding processes, and both bacteria and yeast lack adequate post-translational modification machinery for mammalian-specific N- and O-linked glycosylation, gamma-carboxylation, and proteolytic processing. Cell culture systems require high initial capital expenditures, lack scale-up (or down) flexibility, use large volumes of culture media, and often result in relatively low yields. Transgenic livestock can be maintained and scaled-up in relatively inexpensive facilities, use cheap raw materials (animal feed), and can achieve impressive yields of recombinant proteins. Limitations of the transgenic expression systems are related to time-lines and the potential adverse effects of bio-active heterologous proteins on the health of the production animals. In addition, although transgenic expression systems are able to perform complex N- and O-linked glycosylation and gamma-carboxylation, there are species- and tissue-specific characteristics for these modifications that may affect the appropriateness of a given system for the expression of specific proteins or class of proteins (see Section 2.2.3). This latter situation is also an issue with mammalian cell culture.

To target a recombinant protein to the milk of a transgenic animal (Fig. 1), an expression vector containing a gene encoding the protein of interest fused to milk-specific regulatory elements is usually introduced in the germline of the chosen production species. Pronuclear microinjection of one-cell embryos or, alternatively, transfection into a primary cell population suitable for somatic cell nuclear transfer have both been used to generate transgenic founders. Following integration into the germline, the mammary gland-specific transgenes are predictably inherited by the offspring of the founder animal. Often, transgenic animals will express the protein(s) of interest at concentrations surpassing 2 mg/ml (Table 1) depending on the mammary-specific regulatory sequences, the gene expressed, and the integration site of the transgene. Milk can easily be obtained using established large-scale technologies of the dairy industry, and is an excellent starting material from which recombinant therapeutic proteins can be purified.

2.2. Production species

The choice of the production species is largely driven by the expected quantity of the therapeutic protein needed. As summarized in Table 2, there is usually a trade-off between milk yield and time to natural lactation. Another consideration may be a

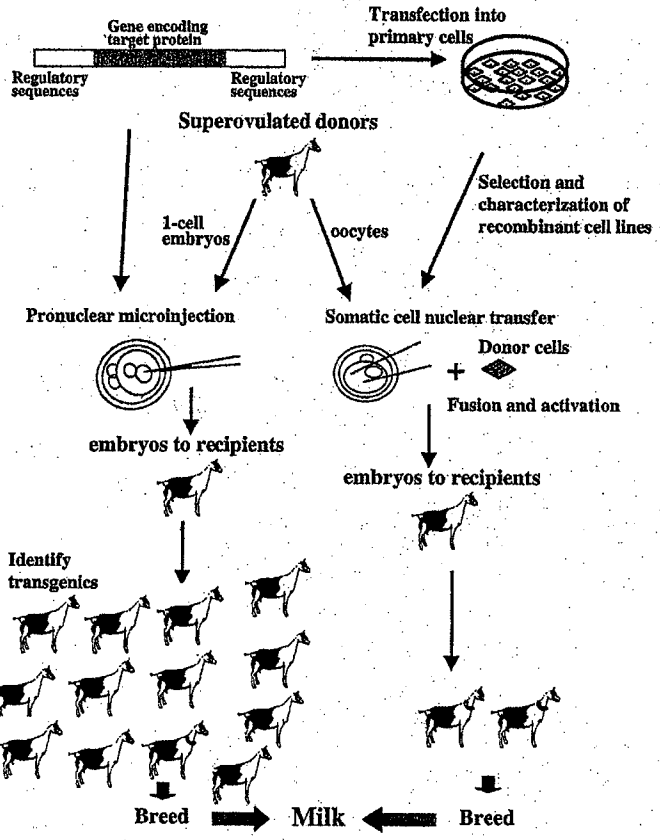

Fig. 1. Schematic representation of the transgenic production process.

species-specific ability to perform specialized post-translational modifications more efficiently.

2.2.1. Mice

Transgenic mice are mainly used for the testing of expression constructs prior to or concomitant with the generation of larger founder transgenic animals. The milk promoter sequences from all mammals tested appear capable of expression in the mouse mammary gland [6,7,9,10]. This allows the relatively inexpensive and rapid evaluation and optimization of transgene constructs. The access to this rapid, reliable test system has proven crucial to the development of milk expression technology, allowing the definition of regulatory sequences that efficiently target expression of heterologous genes to the mammary gland (reviewed in Ref. [10]). Transgenic mice can also provide an early warning of potential adverse effects on the health of animals caused by the production of the recombinant protein in milk.

Table 1

Summary of published data reporting the expression of recombinant proteins in the milk of transgenic large animals. Expression of human growth hormone and human factor IX in the milk of transgenic goats was obtained by introducing the transgene directly into the mammary gland using a "gene therapy" approach

Species	Therapeutic protein	Regulatory sequences	Maximum expression	References
Pig	Human protein C	Mouse WAP	2 mg/ml	[19]
	Human factor VIII	Mouse WAP	2.7 μg/ml	[18]
	Human factor IX	Mouse WAP	0.2 mg/ml	[19]
Sheep	Human α_1-antitrypsin	Sheep β-lactoglobulin	37 mg/ml	[6]
	Human factor IX	Sheep β-lactoglobulin	0.025 ng/ml	[6]
	Human fibrinogen	Sheep β-lactoglobulin	5 mg/ml	[6]
	Human protein C	Sheep β-lactoglobulin	0.3 mg/ml	[6]
	Human factor VIII	Sheep β-lactoglobulin	4-6 ng/ml	[58]
Goat	Human long-acting tPA	Goat β-casein	8 mg/ml	[7]
	Human antithrombin	Goat β-casein	14 mg/ml	[7]
	Human α_1-proteinase inh.	Goat β-casein	20 mg/ml	[7]
	Anti-Lewis Y IgG1	Goat β-casein	14 mg/ml	[8]
	Human G-CSF	Goat β-casein	50 μg/ml	[59]
	Human growth hormone	MoMLV LTR	118 ng/ml	[48]
	Human factor IX	Bovine β-casein	13. 7 ng/ml	[49]
Cattle	Human lactoferrin	Bovine α_{S1}-casein	3.5 mg/ml	[25]
	Human α-lactalbumin	Human α-lactalbumin	2.4 mg/ml	[6]
	Human serum albumin	Goat β-casein	48 mg/ml	[26]

Table 2

Summary of relevant reproductive and lactation characteristics of species used in the transgenic production of recombinant proteins. The data represent average numbers and usually apply to dairy breeds for the livestock species. There are often wide variations according to breed (or strain) within a species

Species	Reproductive maturity (months)	Length of gestation (months)	Average number of offspring	Average% of transgenic birth per total offspring	Average yield per natural lactation (l)	Total protein content in the milk (%)[a]
Mouse	1	0.75	10	10–20	0.0015	9.0
Rabbit	6	1	8	5–15	1.5	13.9
Pig	8	4	9	5–15	80–120	4.8
Sheep	8	5	2	3–5	300–500	5.5
Goat	8	5	2	3–5	600–800	2.9
Cattle	15	9	1	0.5–3	8–10,000	3.4

[a]Data taken from Ref. [60].

Obviously, the very limited milk yield from transgenic mice restricts expression of recombinant proteins to milligram amounts. But in some instances, this is sufficient to obtain meaningful data on the therapeutic protein of interest. As an example, it was possible to purify enough Malaria antigen $MSP1_{42}$ from transgenic mouse milk to test for immune protection in a primate model [11].

2.2.2. Rabbits

Transgenic rabbits have several advantages over other transgenic bioreactors: generation of transgenic animals by microinjection is straightforward and inexpensive relative to ruminants; rabbits have a short gestation interval that allows up to 8 lactations/year; and up to 1.5 l of milk can be obtained per lactation, a 1000-fold what can be obtained from mice. Rabbits have been used successfully for the production of gram quantities of proteins [12–15]. Labor-intensive milking and high husbandry costs may limit the use of transgenic rabbits to the production of therapeutic proteins needed in small quantities. One example of such an indication is the rare and severe lysosomal storage disorder, Pompe disease. In the severe infantile cases of this disease, characterized by a deficiency of acid α-glucosidase needed for the degradation of lysosomal glycogen, patients do not survive beyond the age of one year. In a small trial, recombinant α-glucosidase produced in the milk of transgenic rabbits was used to treat four young patients [16], stabilizing what would normally be a rapidly declining clinical condition. This trial indicated that protein therapy could be beneficial for Pompe disease patients. However, scale-up of the rabbit production system for α-glucosidase may still be problematic, and other systems (or production animals) with higher yields may be more adequate for the larger patient population.

2.2.3. Pigs

The production in the milk of transgenic sows of human Protein C [17], factor VIII [18], and factor IX [19] has been reported. Although pigs are not ordinarily viewed as dairy animals, a surprising amount of milk (100–200 liters) can be obtained from a lactating sow. With these production levels, pigs could be useful for the production of some of the vitamin K-dependent proteins such as factor IX and Protein C, which have estimated needs in the low kilogram range. Interestingly, pig mammary gland cells appear to carry out the complex post-translational modifications (γ-carboxylation, proteolytic processing) on factor IX and Protein C at rates higher than those encountered with mammalian cell and transgenic mouse milk systems [20].

2.2.4. Sheep

The first published report of production of therapeutic proteins in the milk of transgenic dairy farm animals was the targeting of factor IX (0.025 ng/ml) and α_1-antitrypsin (5 µg/ml) to the milk of transgenic ewes [21]. More recently, other proteins such as fibrinogen and factor VIII have also been expressed in the mammary gland of transgenic sheep (Table 1). The most extensively characterized of all sheep milk-expressed recombinant proteins is α_1-antitrypsin. An expression level in excess of 30 g/l was achieved in the milk of a founder transgenic ewe [6]. This was obtained using a chimeric transgene, which consisted of a genomic minigene encoding α_1-antitrypsin fused to the sheep β-lactoglobulin promoter sequences. The transgene integration in this particular line proved unstable, and another transgenic sheep line was identified from which heterozygous females produced α_1-antitrypsin at levels of 13–16 g/l [6]. A herd generated from this line has been propagated, producing a large amount of purified material for use in clinical trials [6].

Aerosolized recombinant α_1-antitrypsin derived from sheep milk is currently in phase II trials for both the cystic fibrosis and the hereditary emphysema indication (PPL Therapeutics Inc., [22])

2.2.5. Goats

A number of recombinant therapeutic proteins has been expressed in transgenic goats (Table 1). Dairy goats, with an average milk output per doe on the order of 600–800 liters per natural lactation, are very well adapted to the production of therapeutic proteins. Concentrations of heterologous therapeutic proteins in excess of 5 g/l of milk have been achieved reproducibly. The time-line from initiation of transgene transfer to natural lactation of resulting transgenic does is 16–18 months for goats. A large number of production females can be easily generated from a transgenic male using standard artificial insemination or embryo transfer techniques. Relatively small herds of a few hundred transgenic does can then easily yield several hundreds of kilograms of purified product per year. This level of production can meet the manufacturing needs of the large number of recombinant antibodies currently in development, several having been expressed in the milk of transgenic goats [8].

Of all recombinant proteins produced in transgenic expression systems, milk-derived antithrombin has gone farthest down the path of regulatory approval [23]. Clinical trial material was obtained using a process that achieves greater than 99.99% purity, with a cumulative yield in excess of 50% [24]. Structurally, the recombinant human antithrombin derived from transgenic goat milk was indistinguishable from antithrombin obtained from human plasma fractionation, with the exception of the carbohydrates. However, the milk-derived antithrombin showed equivalent bioactivity in various pre-clinical and clinical situations [23]. Two phase-III clinical trials, evaluating the effects of recombinant antithrombin in treating heparin resistance in cardiac surgical patients, have been completed, attaining their primary endpoint (Genzyme Transgenics Corp., [24]). Further clinical development of recombinant antithrombin is currently underway.

2.2.6. Cows

In terms of transgenic production, cattle have important advantages and one major drawback. Dairy cows have a yearly milk output in the range of 10,000 liters; consequently, with concentrations routinely achieved with most mammary gland-specific proteins, yields of tens of kilograms of recombinant proteins can be produced by one lactating transgenic cow. In addition, due to the economic importance of bovine agriculture, embryo culture and transfer technologies are well established for cattle breeds. This, combined with the large availability of oocytes derived from abattoirs, allows for the efficient generation of transgenic cows by somatic cell nuclear transfer. However, with cattle it takes almost 3 years from the onset of transgene transfer to obtain milk from a natural lactation. Strategies aiming at reducing this interval will probably need to be implemented for most projects. For example, somatic cell nuclear transfer to generate several identical founder animals combined with lactation induction, can significantly increase the speed at which large

amounts of recombinant proteins are available [25,26]. Calves can be hormonally induced to lactate as early as 2 to 3 months after birth, making it possible to obtain recombinant proteins approximately one year after embryo transfer. Since 6–8-month-old calves can produce several liters of milk per day following hormonal induction (Echelard *et al.*, unpublished data), it is theoretically possible to start generating early clinical trial material less than 18 months after embryo transfer. Although these time-lines are not as short as normally achieved with mammalian cell culture, the tremendous scale-up potential offered by transgenic cattle may compensate for this drawback, especially for indications that necessitate large quantities of protein.

2.3. Engineering of mammary gland-specific transgenes

Transgenes that target the expression of heterologous proteins to the mammary gland are usually constructed by fusing the gene encoding the target protein to regulatory sequences of highly expressed milk-specific proteins. Transgenes (reviewed in Refs. [7,10]) have been constructed with the regulatory sequences of: the mouse- and rabbit-WAP genes; sheep β-lactoglobulin; goat, guinea pig, and bovine α-lactalbumin; rat, rabbit, goat, and bovine β-casein; and bovine-alpha$_{S1}$ casein. Depending on the regulatory sequences employed and the architecture of the target gene, varying levels of expression of the protein of interest were observed (see Table 1). In general, for the same gene, genomic sequences were expressed at higher levels than cDNAs [27,25], although the presence of regulatory elements within the introns of the target gene can be counter-productive in some instances [28].

The success of the transgenic production approach is dependent on high-level, well-controlled expression of the heterologous gene. The design of the transgene is crucial. It is particularly important to remove extraneous enhancers that may interfere with expression of the transgene in the mammary gland, or lead to undesirable ectopic expression. Cryptic splice sites have also been shown to have potentially deleterious effects on the RNA level of the target gene [29]. The mechanisms that govern the silencing of an artificially constructed chimeric transgene are not always well understood. Particularly useful is the ability to verify that a specific combination of mammary gland regulatory sequences and heterologous gene will lead to high-level secretion of the target protein in milk in the mouse system, in advance of generating transgenic large animals.

Since the generation of large transgenic animals can be technically challenging and very costly, efforts have been expanded on strategies aiming at decreasing position-effect sensitivity of transgenes to increase the proportion of productive lines. One strategy is to design very large transgenes using yeast [30] and bacterial artificial chromosomes [31], insuring the presence of most mammary gland-specific regulatory elements and shielding the transgene transcription unit from the surrounding chromatin. One drawback is that precise insertion of the foreign gene in such constructs may be challenging and that the co-expression of several proteins, as for the production of recombinant antibodies, may be impractical. Another strategy is to supply compact, well-defined insulator elements (see Chapter 11) to the transgene.

Experiments in transgenic Drosophila [32], as well as in transgenic mice [33,34], have shown that flanking a transgene with the chicken β-globin 5′cHS4 control element can significantly reduce silencing due to chromosomal position effects and promoter interference. We have used the chicken 5′cHS4 β-globin insulator sequence to shield the mammary-expression cassette from the surrounding chromatin and from the neomycin resistance cassette in constructs used to derive cows by somatic cell nuclear transfer [26].

2.4. Generation of transgenic animals

A number of approaches have been investigated for the development of transgenic animals. However, the great majority of transgenic large animal bioreactors have been derived either by pronuclear microinjection or, more recently, by somatic cell nuclear transfer. Other techniques are in development [35], and may eventually facilitate further transgenic modifications. An alternative approach is to introduce transgenes transiently in the mammary glands of lactating animals.

2.4.1. Pronuclear microinjection

To date, pronuclear microinjection is the method employed to produce most of the transgenic animal bioreactors. Direct microinjection of foreign genes into pronuclei was first reported for mice [1] and then adapted to gene transfer into rabbits, pigs and ruminants [2,36,37]. In general, fertilized eggs are collected from superovulated donors and at least one pronucleus is microinjected with a diluted DNA solution, introducing a few hundred copies of linear transgene(s) per embryo. This is followed by either uterine or oviductal transfer to a recipient, depending on the extent of *in vitro* culture after microinjection. After transfer, manipulated embryos are allowed to develop to term, and offspring are then screened for the presence of the transgene(s). Although this method is technically simple, the main challenge is to devise an efficient embryo collection and transfer system for the chosen species.

While successful, the technique of pronuclear microinjection has limitations. The frequency of transgene integration into the genome of large animals is low, with less than 0.1% (cattle) to up to 2% (pigs) of the microinjected embryos producing transgenic offspring [2,6,7,9,12,21,25,35–37]. Transgene integration into the genome is a random process, and the number of integrations, their position in the genome, and the number of transgene copies per cell, are unpredictable. The frequent generation of mosaic founder animals, carrying the transgene only in some of their cells, is another problem associated with pronuclear microinjection (reviewed in [38]).

2.4.2. Somatic cell nuclear transfer

The demonstration in sheep [39], cattle [40], goats [41], pigs [42,43], and rabbits [44] that differentiated somatic cells can function as karyoplast donors in nuclear transfer has expanded the range of possibilities for germline modification in transgenic livestock. Transgenes can be introduced into primary cell lines by transfection

methods such as lipofection or electroporation. This allows for the characterization of the transgenic cell line before the generation of transgenic embryos. Recombinant primary cell lines can be screened for transgene copy-number, integrity of the transgene, and even chromosome localization [45]. Another advantage is the absence of transgene mosaicism, as all animals generated by somatic cell nuclear transfer should be fully transgenic, unless the primary cell line from which they are derived is not pure.

One issue with nuclear transfer is that it is more technically complex than microinjection. Appropriate primary cell culture and transfection conditions must be determined and there are infinite variations on karyoplast/cytoplast fusion and activation conditions. However, the main problem associated with somatic cell nuclear transfer is that, in contrast with microinjection, it is associated with the generation of a high proportion of animals suffering from various developmental abnormalities [46]. Despite these drawbacks, it is clear that for some systems, somatic cell nuclear transfer is more efficient. For example, in the generation of transgenic cattle for the production of recombinant human serum albumin, our group obtained 16 healthy founder calves by nuclear transfer [26], as compared to one transgenic calf by microinjection [47].

2.4.3. Transient approaches

Inspired by somatic cell gene therapy approaches, these strategies aim to bypass the germline in obtaining recombinant protein production in animals. In an early attempt, the mammary glands of goats hormonally induced to lactate were infected with replication-defective retroviral vectors carrying the human growth hormone (hGH) cDNA regulated by the Moloney murine leukemia virus (MoMLV) long terminal repeat (LTR) [48]. Initial expression levels of up to 118 ng of hGH/ml of milk stabilized, by day 3, to 3–12 ng/ml. Another group [49] directly introduced liposome-plasmid complexes into the mammary gland of lactating goats. In these constructs, the factor IX gene was regulated by bovine β-casein sequences. Production of human factor IX was 13.7 ng/ml of goat milk at day 3, with the majority of the recombinant factor IX active and correctly γ-carboxylated.

The advantage of the transient methods is that expression of the recombinant protein is obtained quickly, without the delay caused by the generation interval required to produce and breed transgenic animals. Moreover, the ability to quickly generate gram amounts of a recombinant protein in the mammary gland system would be invaluable. This material could be used to predict post-translational modifications of a specific protein in the prospective large-scale bioreactor, and possibly to generate pre-clinical and early-clinical phase data. The simplicity of the system could allow relatively cost-effective production of small amounts of many potential protein drug candidates and facilitate the evaluation of each drug. However, the production levels reported have been uniformly very low. This would preclude manufacturing use of this approach until a significant increase (at least 100-fold) in expression levels is achieved.

2.5. Protein purification from the milk of transgenic animals

The usual challenge of recombinant protein purification from mammalian cell culture is that high volumes of media must be processed due to low expression levels of the protein (often in the mg/l range). Typical contaminants in serum-free media are cell debris, lipids, host DNA, host cell proteins, viruses and pyrogens. In serum-supplemented cultures, other major impurities include bovine serum albumin, transferrin, and bovine IgG.

Recombinant proteins produced in the milk of transgenic goats at the relatively modest level of 2 g/l represent 7% of the total protein. Since recombinant proteins are usually expressed as whey proteins, the initial separation steps aim to achieve casein-free, fat-free and lactose-free protein concentrates in which the recombinant protein is at 30–60% purity, depending on the expression level. Fat may be separated from milk using standard centrifugal procedures or by membrane filtration [6–8,24]. Membrane filtration can also be used to eliminate most of the caseins and small molecules. The resulting clarified whey is an excellent starting material for chromatographic purification.

Several groups [6,24] have described purification processes for the isolation of clinical grade therapeutic proteins from the milk of transgenic large animals. For the purification of recombinant antithrombin from goat milk, the following steps result in a product with purity greater than 99.99%: clarification through a 500-kd tangential flow membrane filtration unit, capture and then elution from a heparin affinity chromatography column, anion-exchange chromatography and hydrophobic interaction chromatography [24]. The cumulative yield, after a number of production runs, was greater than 55% and there were no detectable contaminating proteins.

3. Other transgenic bioreactors

3.1. Production of recombinant proteins in the blood of transgenic animals

Fractionation of human and animal plasma is a well-established technology. In addition, porcine factor VIII has been used extensively for the treatment of hemophiliacs who develop inhibitors to human Factor VIII. Although blood collection is more invasive than milking, it can be applied several times during the lifespan of the production animal. These factors, and the availability of well-characterized blood-specific regulatory sequences, have led to the development of transgenic animals that produce recombinant proteins in their blood.

In 1992, the production of recombinant human hemoglobin A (HbA) was reported in transgenic pigs [50] carrying a transgene composed of the human β-globin locus control region linked to the human α- and β-globin genes. The objective was to produce recombinant human hemoglobin to be used as a cell-free oxygen carrying red-blood cell substitute. Levels of up to 9% human HbA dimers (human α/human β) were formed with the remainder of the hemoglobin being

composed of hybrid dimers (12% human α/pig β) and of fully pig dimers (79%). In a follow-up report [51], a higher expression level of human hemoglobin (up to 24% human dimers) was achieved. This corresponds to 32 g of human hemoglobin per liter of transgenic pig blood. The improvement in expression appeared to be related to the use of the porcine β-globin promoter to drive the human β-globin gene. Animals appeared to tolerate well the high levels of human HbA in their erythrocytes. Structural and functional investigations of recombinant human HbA isolated from the erythrocytes of transgenic pigs confirmed correct expression, post-translational processing, and assembly of human HbA [52]. However, even with this high expression level, purification of human hemoglobin from the hybrid and pig hemoglobin may be too costly. With the advent of porcine somatic cell nuclear transfer, a strategy aiming at generating transgenic pigs producing solely human hemoglobin is now attainable, potentially decreasing production costs.

Another potential use of the recombinant hemoglobin expression system takes advantage of the property of terminally extended α-globin to remain functional. Carboxy-terminal fusions of human α-globin with α-endorphin and magainin were expressed in the erythrocytes of transgenic mice [53]. Fusion proteins were expressed at levels corresponding to 25% of the total hemoglobin, seemingly without ill effects on the transgenic mice. This strategy could become an alternative to the chemical approach for the large-scale synthesis of therapeutic peptides, especially if cost-efficient methods to cleave away the peptide of interest from the fusion protein were to be devised.

3.2. Transgenic chickens

The concept of targeting the expression of foreign proteins to the eggs of transgenic chickens has generated considerable enthusiasm worldwide, as judged by the large number of biotechnology companies engaged in research towards that goal. The total US egg production surpassed 85 billion in 2001 (National Agricultural Statistics Service, Agricultural Statistics Board, U.S. Department of Agriculture). It is easy to understand that even with transgenes that would allow expression of only a few hundreds of milligrams of therapeutic proteins per egg, transgenic chickens could be scaled up to commercial levels. Technologies for the manipulation of egg by-products are available, and vaccines, often injected into millions of people, are routinely produced from chicken eggs. Purification of proteins for pharmaceutical applications should then be readily achievable in this system. Another potential advantage, if tightly regulated egg-specific regulatory sequences can be identified, would be the possibility of producing very bio-active molecules such as insulin or erythropoietin without affecting the health of the transgenic hens.

At the time this chapter was completed, only one peer-reviewed article describing the production and yield of heterologous proteins in the eggs of transgenic hens has appeared [54]. One explanation is the relative inaccessibility of chicken embryos. Production of germline-transmitting transgenic chickens has been technically

challenging. Techniques such as direct microinjection, stem cell- and primordial germ cell-based strategies, somatic cell nuclear transfer, sperm-mediated transgenesis, and viral-based methods have all been applied to chicken, and some progress has been reported [55,36]. In the only report on transgenic production in eggs [54], transgenic chickens were generated using replication-deficient retroviral vectors (derived from the avian leukosis virus) carrying the β-lactamase marker gene under the control of the CMV promoter. Expression of concentrations of up to 1.34 μg/ml of β-lactamase was recorded in egg whites over several generations. Predictably, due to the non-specific nature of the CMV regulatory sequences, even higher levels of β-lactamase (up to 6.7 μg/ml) were observed in the serum of transgenic hens. Once regulatory sequences that can efficiently target the expression of heterologous genes to the egg are discovered, commercially viable levels of recombinant proteins should be achievable.

3.3. Bladder-specific expression

Although the bladder does not come to mind as an obvious source of recombinant proteins, urine has long been used as a source of biologicals. For example, FSH purified from the urine of post-menopausal women is still used clinically for the treatment of anovulation and in assisted reproduction technologies such as *in vitro* fertilization (IVF) and intra-cytoplasmic sperm injection (ICSI). Additionally, several drug preparations used in estrogen replacement therapy are isolated from pregnant mare urine. In 1998, one group [56] described the use of the mouse uroplakin II (a bladder-specific gene) promoter to target the expression of hGH to the urine of transgenic mice. Low expression levels (up to 0.5 μg/ml) were observed. At these levels, commercial transgenic production of recombinant proteins would be impractical. If it were possible to significantly increase protein expression levels, the bladder-specific system may have the advantages that urine can be harvested soon after birth and throughout the life of the animal, and that urine is a less complex medium than milk or blood, potentially decreasing purification costs. However, it is not clear that it is physiologically possible to increase the protein secretion characteristics of the urothelium cells, nor that it would be compatible with the health of the producing animals.

3.4. Seminal vesicle

The potential of the seminal vesicle as a source for recombinant proteins was examined by a group that produced transgenic mice using a chimeric construct linking the murine P12 promoter to the hGH gene [57]. One line was found to express up to 0.5 mg of hGH/ml of pre-ejaculatory fluid obtained from seminal vesicles of sacrificed transgenic males. Application of this technology to pigs which can be ejaculated 2–3 times per week (200–300 ml per ejaculate), could lead to the production of several grams of recombinant protein per year.

4. Conclusions

Expression of recombinant proteins by transgenic animals represents an opportunity to achieve cost-effective, large-scale production of a wide variety of therapeutics. During the last 15 years we have seen the evolution of mammary-gland specific production from proof-of-concept to late clinical stage. At this juncture, it is clear that the mammary gland expression system can be used to produce large quantities of highly purified, appropriately modified complex proteins. The clinical efficacy of the proteins furthest along in clinical trials, i.e., antithrombin and α_1-antitrypsin, will determine regulatory approval, rather than issues related to the recombinant production system. Other transgenic production systems, such as transgenic chickens and blood-derived proteins, appear very promising but have not yet overcome basic technical issues or produced compounds in human clinical trials.

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